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pdx-1 function is specifically required in embryonic β cells to generate appropriate numbers of endocrine cell types and maintain glucose homeostasis

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Abstract

The *pdx1* gene is essential for pancreatic organogenesis in humans and mice; *pdx1* mutations have been identified in human diabetic patients. Specific inactivation of *pdx1* in adult β cells revealed that this gene is required for maintenance of mature β cell function. In the following study, a Cre-lox strategy was used to remove *pdx1* function specifically from embryonic β cells beginning at late-gestation, prior to islet formation. Animals in which *pdx1* is lost in insulin-producing cells during embryogenesis had elevated blood glucose levels at birth and were overtly diabetic by weaning. Neonatal and adult mutant islets showed a dramatic reduction in the number of insulin$^+$ cells and an increase in both glucagon$^+$ and somatostatin$^+$ cells. Lineage tracing revealed that excess glucagon$^+$ and somatostatin$^+$ cells did not arise by interconversion of endocrine cell types. Examination of mutant islets revealed a decrease in proliferation of insulin-producing cells just before birth and a concomitant increase in proliferation of glucagon-producing cells. We propose that *pdx1* is required for proliferation and function of the β cells generated at late gestation, and that one function of normal β cells is to inhibit the proliferation of other islet cell types, resulting in the appropriate numbers of the different endocrine cell types.

Keywords: Pancreas; Islet; Diabetes; Cre-lox; Lineage tracing

Introduction

Mature pancreatic islets are composed of five different endocrine cell types (α: glucagon, β: insulin, δ: somatostatin, ε: ghrelin, and PP: pancreatic polypeptide) arranged in a typical architecture where the β cells, which make up the large majority, are found at the core and the other cell types are located at the periphery or mantle. Pancreatic endocrine cells appear in two waves during mouse pancreatic organogenesis (Pictet et al., 1972; Pang et al., 1994). During the first wave, which begins at embryonic day 9.5 (e9.5), the glucagon- and insulin-expressing cells that arise differ in their gene expression pattern from the mature cells found in adult islets. First wave glucagon-producing cells express pro-hormone convertase 1/3 (PC1/3) rather than PC2, which is expressed by mature α and β cells (Lee et al., 1999; Wilson et al., 2002), and first wave insulin-producing cells lack the glucose transporter, GLUT2, a marker of mature β cells (Pang et al., 1994). Insulin/glucagon double-positive cells have been detected at early developmental stages and have been postulated to represent an intermediate stage of endocrine differentiation (Alpert et al., 1988; Teitelman et al., 1993). Lineage tracing studies, however, suggest that these cells

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do not in fact give rise to α or β cells of mature islets (Herrera, et al., 1994; Herrera, 2000). Thus, the function of these first wave single and double hormone-positive cells remains unclear.

The second wave of endocrine differentiation occurs between e13.5 and 16.5, and is believed to produce the cells of the mature islets (Pictet et al., 1972; Pang et al., 1994). The mechanism driving the relatively sudden increase in endocrine cell formation at this particular time in development is unknown, although signals including the Notch/Ngn3 pathway that affect precursor proliferation and differentiation are likely to be involved (Apelqvist et al., 1999; Murtaugh et al., 2003; Jensen, 2004). At late gestation and early postnatal stages, the islets begin to adopt their characteristic core/mantle architecture. Little is known about how the different endocrine cell types or their precursors interact with one another to generate the correct proportions of the various cell types. Several studies suggest that early glucagon-producing cells are required specifically for the generation of first wave insulin-producing cells (Dohrmann et al., 2000; Prasadan et al., 2002; Vincent et al., 2003), but it is uncertain whether similar cell–cell interactions are involved in generating endocrine cells at the secondary transition.

Reverse genetic studies in mice are helping to elucidate the complex pathways and gene interactions involved in pancreas development (Wilson et al., 2003). Genetic or cell deletion studies in the absence of lineage tracing techniques can, however, result in difficulties in deciphering cell differentiation pathways. Loss of one cell population in a particular mutant condition may have a profound non-cell autonomous effect on another cell population. For example, selective deletion of PP-producing cells, which results in the loss of both insulin- and somatostatin-producing cells, could be explained either by a PP-expressing precursor cell population giving rise to these two cell types, or by PP-expressing cells producing an inductive signal required for the production of β and δ cells (Herrera et al., 1994). Lineage tracing analyses have helped distinguish between these possibilities, and suggest that mature β cells do indeed arise from cells that previously expressed PP (Herrera, 2000).

The homeobox gene *pdx1* is expressed within the developing pancreatic endoderm in all vertebrates so far examined (Gannon and Wright, 1999). In the mouse, *pdx1* expression begins at e8.0 (Guz et al., 1995; Li et al., 1999), prior to the onset of pancreatic bud formation and islet hormone gene expression, and is initially detected throughout the pancreatic epithelium. By late gestation, *pdx1* expression is selectively maintained at high levels in β cells, with low levels of expression in acinar cells (Guz et al., 1995; Wu et al., 1997). Loss of *pdx1* function results in an early block in pancreatic outgrowth and differentiation in both mice and humans (Jonsson et al., 1994; Offield et al., 1996; Stoffers et al., 1997a,b). The pancreatic rudiment of *pdx1* null mouse embryos does contain transient, first wave insulin$^+$ cells (Ahlgren et al., 1996), and longer-lived glucagon$^+$ cells (Offield et al., 1996), indicating that *pdx1* is not required to generate first wave endocrine cells. In addition to an early role in pancreatic bud outgrowth, studies using tetracycline-inducible *pdx1* inactivation demonstrated that *pdx1* is also specifically required between e11.5 and e13.5 in order for subsequent differentiation of endocrine and exocrine cells (Holland et al., 2002).

Mice heterozygous for a *pdx1* deficiency are glucose-intolerant (Ahlgren et al., 1998; Dutta et al., 1998; Brissova et al., 2002), consistent with the finding that humans carrying dominant *pdx1* mutations are predisposed to a form of Type 2 diabetes called maturity onset diabetes of the young type 4 (MODY4) (Stoffers et al., 1997a,b, 1998; Macfarlane et al., 2000). The continued essential role for *pdx1* in mature β cells (Ahlgren et al., 1998; Holland et al., 2002) fits well with its identification as a direct activator of several β cell-specific genes that control glucose utilization and metabolism, including insulin, IAPP, glucokinase, *Pax4*, and *pdx1* itself (Chakrabarti et al., 2002; Cissell et al., 2002).

Direct evidence that *pdx1* is essential for maintaining mature β cell function comes from studies using tetracycline-inducible *pdx1* inactivation in adult mice (Holland et al., 2002) as well as conditional inactivation studies using an insulin promoter-driven Cre transgene that resulted in a loss of Pdx1 protein between 3 and 5 weeks after birth (Ahlgren et al., 1998). This mature β cell-specific loss of *pdx1* caused a dramatic decrease in insulin, Nkx6.1, and GLUT2 expression, a concomitant increase in the number of glucagon$^+$ cells, and overt diabetes in 3–5 month old mice. The excess glucagon$^+$ cells and large number of insulin/glucagon co-producing cells that were detected in the islets of these mice led to the suggestion that insulin$^+$ cells acquire glucagon expression after removal of the repressive influences of Pdx1. In the absence of lineage tracing, it is impossible to determine the origin of the excess glucagon$^+$ cells. One can envisage several ways in which such cells could arise from β cells or an insulin-expressing precursor cell type: (1) mature β cells de-differentiate to a more immature glucagon/insulin co-expressing cell type; (2) β cells slowly trans-differentiate towards a mature α cell type; or, (3) β cells loss promotes generation of new endocrine cells from an unidentified progenitor cell, which then gives rise to insulin/glucagon double-positive cells.

In summary, therefore, while *pdx1* is critical early in pancreas development for global organ formation and differentiation, as well as later in mature β cells, it is unclear what role it plays at the secondary transition in generating the β cells that will contribute to mature islets. We report here the results of a Cre-lox conditional inactivation study that provides details on *pdx1* function early in the definitive β cell lineage (during the second wave of endocrine differentiation), in which we assessed the consequences of *pdx1* inactivation by including lineage-tracing analysis. In this study, we used an optimized rat insulin promoter-Cre transgenic line (RIP-Cre; Postic et al., 1999; Gannon et al., 2000a,b,c) which shows functional recombination as early as e11.5 in developing pancreas and efficient (>85% of β cells) excision of *loxP*-flanked DNA (Postic et al., 1999; Gannon et al., 2000c). We demonstrate that loss of *pdx1* from early β cells leads to a severe reduction in the number of insulin$^+$ cells beginning at late gestation stages. β cell-specific inactivation of *pdx1* during embryogenesis results in early-onset diabetes, disrupted islet architecture, and increased numbers of glucagon$^+$ and somatostatin$^+$ cells at the expense

of β cells. The lineage-tracing analysis allowed tracking of the fate of cells that activated the RIP-Cre transgene. We provide evidence for a linkage between the overall β cell number and the mechanisms that control the proliferation/differentiation of glucagon$^+$ cells.

Materials and methods

Construction of floxed pdx1 allele and generation of mice

The *pdx1*$^{tm4(E2)Cvw\text{-}TKneo}$ targeting construct (see Fig. 1) was generated using the pLox-TKneo vector (Orban et al., 1992). Standard subcloning methods were used to introduce *loxP* sites into the intron and just after the poly-A addition site, thus flanking the DNA binding domain-containing exon 2 (Fig. 1; details available upon request). The integrity of *pdx1* sequences and *loxP* sites was checked by diagnostic restriction enzyme digestion and sequencing. Plasmid DNA was CsCl-purified, the entire insert isolated by low-melt agarose gel electrophoresis after *Not*I digestion, and purified using Gelase (Epicentre Technologies).

200 μg of linearized *pdx1*$^{tm4(E2)Cvw\text{-}TKneo}$ DNA was electroporated into TL1 129 SvEv ES cells by the Vanderbilt University Transgenic/ES Cell Core, and ES cell clones were grown as described (Hogan et al., 1994). Correctly targeted ES cell clones were identified by Southern blot analysis of genomic DNA [*Pst*I or *Bam*HI digest, 500 bp 3′ *pdx1* cDNA internal probe (Figs. 1A, B); *Xba*I digest, 550 bp *Xba*I–*Eco*RI external probe (data not shown)]. On *Pst*I digestion, the 3′ cDNA probe recognizes a 4.0-kb endogenous locus band and a 2.7-kb band for the *loxP*-marked allele. *Bam*HI digestion generates an endogenous allele band of 7.0 kb or an 11.0 kb band from the *loxP*-marked allele. A total of 543 ES cell clones were screened with a targeting efficiency of 6.8%. Thus, 37 correctly targeted clones were identified. The 1B8 ES cell clone containing the correctly targeted *pdx1*$^{tm4(E2)Cvw\text{-}TKneo}$ allele was electroporated with CMV-Cre plasmid to remove the PGK-neo^R/HSV-TK cassettes. Cre-mediated excision of the selection cassette was determined using an 800 bp fragment of the neo^R cassette, which recognizes a 1.0 kb *Pst*I band. The presence of exon two was confirmed by retention of the 2.7 kb *Pst*I band using the 3′ cDNA probe. One clone undergoing the desired recombination event (1B8/10) was injected into C57BL/6 blastocysts to generate chimeric mice. Male chimeras were bred to Black Swiss females and agouti offspring were genotyped by Southern analysis for transmission of the *pdx1*$^{tm4(E2)Cvw}$ allele (*Pst*I digestion; 3′ *pdx1* cDNA probe) from here on termed *pdx1*^{flE2}. *pdx1*^{flE2} heterozygotes were bred to produce *pdx1*$^{flE2/flE2}$ mice, which are phenotypically normal by several criteria (see Results).

The RIP-Cre line used in these studies has been described (Postic et al., 1999; Gannon et al., 2000c). RIP-Cre transgenics were identified by Southern

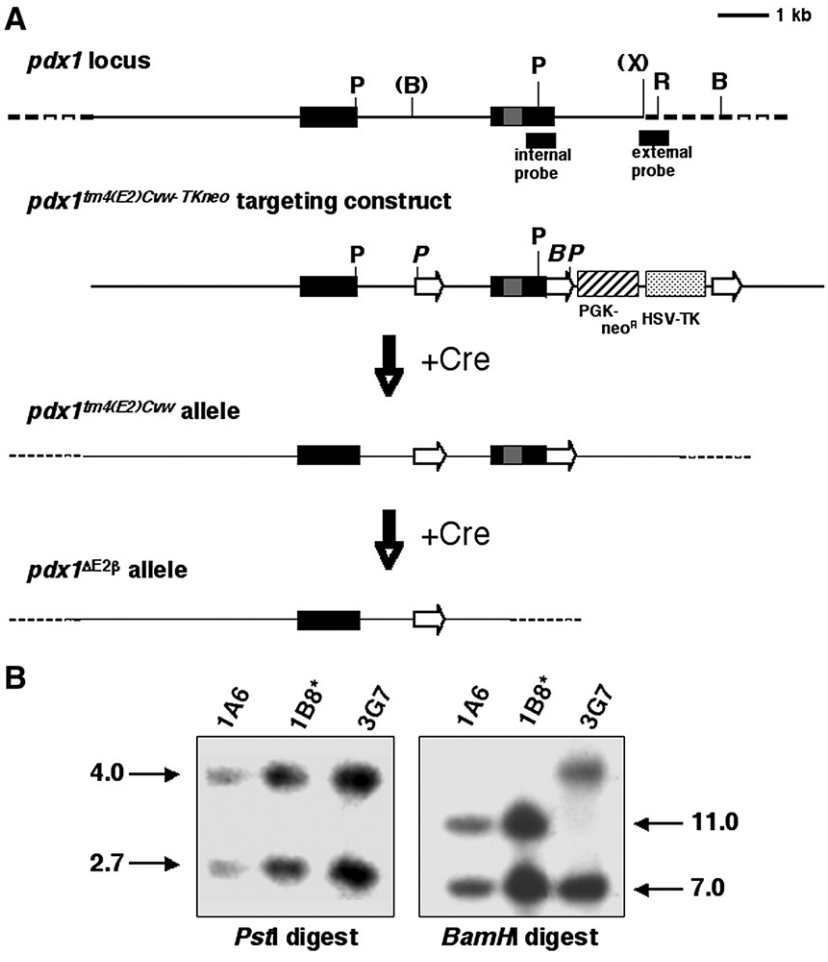

Fig. 1. (A) Diagram of *pdx1* locus, *loxP*-containing targeting construct, and expected products following Cre-mediated recombination. Black boxes: exons, gray box: homeodomain, solid line: genomic regions used in targeting construct, open arrows: *loxP* sites, cross-hatched box: neomycin resistance cassette driven by the PGK promoter, stippled box: thymidine kinase cassette driven by the HSV promoter. Location of internal and external probes for Southern blotting is indicated below the locus. Restriction enzyme sites: B, *Bam*HI; P, *Pst*I; X, *Xba*I. Sites in parentheses are lost in the generation of the targeting construct. Sites in italics are introduced in the targeting construct. (B) Southern blot of DNA from three representative neomycin-resistant ES cell clones using internal probe. *Pst*I digest (left panel): 4.0 kb band, endogenous allele; 2.7 kb band, floxed allele. *Bam*HI digest (right panel): 7.0 kb band, endogenous allele; 11.0 kb band, floxed allele. Asterisk: clone used for Cre electroporation.

analysis (*Eco*RI digestion; 500 bp *Bam*HI–*Cla*I Cre probe). R26R mice, originally from Philippe Soriano (Soriano, 1999), were a gift of Richard Behringer (M.D. Anderson Cancer Center, Houston, TX) and were genotyped by Southern analysis (*Eco*RI digestion; 726 bp *Pvu*I *lac*Z probe). RIP-Cre; R26R bigenic mice were mated with mice heterozygous for the $pdx1^{XBKo}$ null allele generated previously by our laboratory (Offield et al., 1996). We will refer to these mice as $pdx1^{+/-}$. $pdx1^{+/-}$;RIP-Cre;R26R mice were generated at the expected frequency of 1:8. The *pdx1* null allele was identified by Southern blotting (Offield et al., 1996). $pdx1^{flE2/flE2}$ mice were bred to $pdx1^{+/-}$;RIP-Cre; R26R mice to generate $pdx1^{flE2/-}$;RIP-Cre;R26R mice at a frequency of 1:8. $pdx1^{flE2/-}$;RIP-Cre mice will be referred to as $pdx1^{\Delta E2\beta}$. All animals were given water and Lab Diet #5015 mouse pellets *ad lib* on a 12-h light/dark cycle.

Tissue preparation and histology

The morning of the vaginal plug was considered e0.5. Embryonic digestive organs, including pancreata, were dissected in PBS and fixed immediately in ice-cold 4% paraformaldehyde (4 °C; 45 min to 1 h). Tissues were dehydrated in an increasing ethanol series and embedded in paraffin for sectioning. Serial 7 μm sections mounted on glass slides with Sta-on (Surgipath) were used for histology, immunohistochemistry, and immunofluorescence analyses. Analyses were performed on at least five sections from each of at least three separate animals. Frozen tissues were fixed as above, incubated in 30% sucrose overnight at 4 °C, embedded in optimum cutting temperature compound (VWR Scientific, West Chester, PA), and 5 μm sections were cut on a Leica CM 3050 S cryostat.

β-galactosidase detection

Following fixation, tissues were washed twice for 30 min in permeabilization solution (2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet P-40 in PBS). β-galactosidase (β-gal) activity was detected using X-gal as described (Wu et al., 1997; Gannon et al., 2000a,b,c). Tissues were post-fixed and dehydrated for embedding as above, with isopropanol replacing xylene to minimize leaching of the blue precipitate. Images were collected on an Olympus BX41 microscope with an Olympus digital camera and Magnafire program (Optronics, Inc.). Images were all equivalently processed in Adobe Photoshop 6.0.

Immunohistochemistry and immunofluorescence

Sections were deparaffinized in xylene and rehydrated in a descending ethanol series to water. Immunoperoxidase staining for insulin, glucagon, and somatostatin was performed as previously described (Gannon et al., 2000b). Primary antibodies were used at the following dilutions: guinea pig anti-bovine insulin (Linco), 1:1000; rabbit anti-glucagon (Linco), 1:1000; guinea pig anti-glucagon (Linco), 1:500; rabbit anti-human somatostatin (Dako), 1:1000; rabbit anti-Pdx1, 1:25 (Peshavaria et al., 1994); rabbit anti-GLUT2 (a gift of Bernard Thorens, University of Lausanne), 1:500; rabbit anti-phospho-histone H3, 1:50 (Upstate Biotechnology). Two different antibodies against β-galactosidase were utilized: a rabbit anti-β-galactosidase generated against an XlHbox4-β-gal fusion protein that specifically recognizes β-galactosidase on western blot (Gannon et al., 2001), and rabbit anti-β-gal 1:5000 (MP Biomedicals). Antigen retrieval [10 mM citrate buffer, microwave oven 3 min then 1 min at 1000 W, 20 min at room temperature] was performed for immunodetection of Pdx1, GLUT2, phospho-histone H3, and β-gal. Samples were viewed under bright field illumination and photographed with Kodak Ektachrome 64T film, or using an Olympus BX41 microscope and digital camera (Magnafire program). For immunofluorescence, donkey anti-guinea pig CY2 and donkey anti-rabbit CY3 or CY5 were used as secondary antibodies. CY2 was excited at 543 nm, CY3 at 488 nm, and CY5 at 647 nm using an LSM 410 confocal microscope (Zeiss). Optical sections were taken at 1 μm. TIFF images from each experiment were processed equivalently in Adobe Photoshop 6.0.

Glucose tolerance tests (GTT)

Intraperitoneal glucose tolerance tests were performed as described (Gannon et al., 2000b). Following a 16-h fast, baseline blood glucose levels (mg/dl) were measured in 10 μl tail vein blood from mildly anesthetized mice using the Accu-Chek Advantage glucose meter and Accu-Chek test strips (Roche). Glucose (2 mg dextrose/g body weight) in sterile PBS was injected intraperitoneally and blood glucose measured 15, 30, 60, 90 and 120 min after injection. GTT were performed every 2 weeks for 2–4 months beginning at weaning. Insulin radioimmunoassay was performed as previously described (Gannon et al., 2000c).

Results

The floxed pdx1 allele functions as a wild type allele

In order to produce a conditional *pdx1* null allele, *loxP* sites were placed on either side of exon two, which encodes the DNA binding homeodomain. The introduction of the 34 bp *loxP* sequences at these two locations did not interfere with *pdx1* gene function of the floxed allele. $pdx1^{flE2/+}$ and $pdx1^{flE2/flE2}$ animals were born at the expected frequency, survived through adulthood, and were outwardly indistinguishable from control littermates. In neonates and 1-month-old animals, the gross morphology (size and shape) of the pancreata of $pdx1^{flE2/+}$ and $pdx1^{flE2/flE2}$ mice was identical to control littermates, as was islet number, size, and architecture (data not shown). On intraperitoneal glucose tolerance test (IP-GTT), animals of all three genotypes (+/+, flE2/+, flE2/flE2) had a fasting blood glucose level in the normal range (70–150 mg/dl) and efficiently cleared a glucose bolus from the bloodstream, returning to baseline glucose levels within a 2-h period (data not shown). We conclude that the floxed *pdx1* allele is a true conditional null allele with wild type function in the non-recombined configuration.

RIP-Cre mediated pdx1 inactivation occurs during embryonic stages

To inactivate *pdx1* specifically in embryonic pancreatic β cells, mice carrying the floxed *pdx1* allele were bred to mice carrying a RIP-Cre transgene. Inclusion of the R26R reporter allele, in which expression of a *lac*Z reporter cassette depends upon Cre-mediated removal of a *loxP*-flanked "stop" sequence (Soriano, 1999), allowed the detection of cells that have undergone recombination in our experiments. Using R26R mice, we previously characterized the RIP-Cre transgene as being active in isolated pancreatic cells at e11.5 and restricted to insulin-producing cells of the pancreas at all times examined (Gannon et al., 2000c). At P1 and in adults, recombination-based activation of β-gal expression was detected in >85% of insulin-producing cells in the islet core. Expression was never observed in glucagon-expressing cells, acinar cells, or other cells of the foregut.

We reasoned that placing the $pdx1^{flE2}$ allele in *trans* with a *pdx1* null allele (Offield et al., 1996) would require Cre-mediated inactivation of only a single floxed *pdx1* allele, facilitating the generation of *pdx1* null β cells ($pdx1^{\Delta E2\beta}$). To this end, $pdx1^{+/-}$ mice were bred to RIP-Cre;R26R mice (see Methods). Matings between $pdx1^{+/-}$;RIP-Cre;R26R mice and $pdx1^{flE2/flE2}$ mice generated $pdx1^{flE2/-}$;RIP-Cre and $pdx1^{flE2/-}$; RIP-Cre;R26R experimental mice, both at the expected frequency of 1:8. On average, therefore, one quarter of the pups

would be expected to undergo β cell-specific inactivation of *pdx1* (from here called $pdx1^{\Delta E2\beta}$), and half of these pups would also carry the activatable β-gal lineage marker. The phenotypes of both $pdx1^{\Delta E2\beta}$ and $pdx1^{\Delta E2\beta}$;R26R mice were identical, indicating that the presence of the R26R allele had no effect on the function of the Cre transgene, or on the consequences of *pdx1* inactivation. In the subsequent studies, mice of both genotypes were used interchangeably, except for the lineage studies, which required $pdx1^{\Delta E2\beta}$ and R26R in combination.

Based on the detection of RIP-Cre activity by R26R activation, we anticipated that *pdx1* inactivation would occur early in the great majority of insulin-producing cells. The previous analysis of *pdx1* null mutant animals revealed the presence of significant numbers of *pdx1*-independent first wave insulin- and glucagon-positive cells (Ahlgren et al., 1996; Offield et al., 1996). In the current study, pancreata from $pdx1^{\Delta E2\beta}$, $pdx1^{\Delta E2\beta}$;R26R embryos, and control littermates ($pdx1^{+/-}$;RIP-Cre, $pdx1^{flE2/+}$;RIP-Cre, and $pdx1^{flE2/-}$) were examined during the second wave of endocrine differentiation, beginning at e14.5, to assess the effects of Cre-mediated *pdx1* inactivation on the formation of endocrine cells that would go on to contribute to the mature islets.

Initially, we examined embryos at e14.5 to determine whether RIP-Cre mediated recombination resulted in the loss of Pdx1 protein expression from insulin-producing cells. At this time point, the majority of insulin-positive cells should have been generated from the second wave of endocrine differentiation. In sections from control pancreata ($n=7$) at this stage, nuclear Pdx1 protein was detected in 78% of insulin$^+$ cells ($n=132/170$; Fig. 2A), while only 46% of insulin$^+$ cells in the e14.5 conditional mutant ($n=3$) pancreas co-expressed Pdx1 ($n=26/57$; Fig. 2B) indicating that some insulin-producing cells have already undergone recombination and lost Pdx1 expression at this stage. The average number of insulin$^+$ cells per section was identical in both mutant and control pancreata at this stage. At e17.5–e18.5, Pdx1 was detected in 75% of insulin$^+$ cells ($n=280/374$) of control embryos ($n=3$). In contrast, only 19% of insulin$^+$ cells ($n=87/453$) in mutant embryos ($n=4$) had detectable levels of Pdx1 at these stages (data not shown) indicating that recombination of the $pdx1^{flE2}$ allele has occurred in the majority of insulin-producing cells by late gestation. At postnatal day 2, 15% of insulin$^+$ cells found in mutant pancreata still contain detectable levels of Pdx1 protein (data not shown), in agreement with our previously published results showing that this RIP-Cre line mediates recombination in ~85% of β cells (Gannon et al., 2000c).

Since lineage tracing of *pdx1* null mutant cells relies on equivalent efficiencies of recombination for both the $pdx1^{flE2}$ and the R26R alleles, we next wanted to determine whether the dramatic Cre-mediated loss of PDX1 expression seen at e17.5–e18.5 was always associated with activation of the R26R allele. In RIP-Cre;R26R control pancreata ($n=4$ animals) at e18.5, Pdx1 was detected in 90% of recombined (i.e. β-gal$^+$) nuclei (Fig. 2C), consistent with its high level of expression in normal insulin-producing cells (Gamer and Wright, 1995; Guz et al., 1995). In contrast, Pdx1 was not observed in β-gal$^+$ cells in

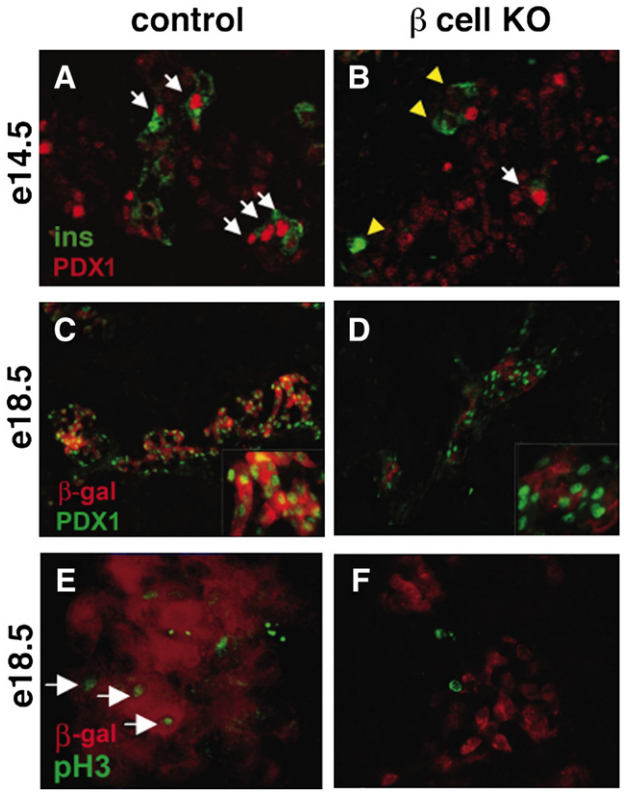

Fig. 2. Loss of PDX1 in insulin-producing cells in $pdx1^{\Delta E2\beta}$;R26R embryos. (A) Sections from control pancreata at e14.5 show intense PDX1 protein expression (red) in the nuclei of most insulin$^+$ cells (green; arrows). (B) In $pdx1^{\Delta E2\beta}$, most insulin$^+$ cells showed no detectable PDX1 protein (yellow arrowheads), although PDX1$^+$/insulin$^+$ cells are present (arrow). Weaker PDX1 expression is observed in ducts and developing acinar cells in all embryos. (C) PDX1 protein is apparent in most nuclei of β-gal expressing (i.e., recombined/red) β cells of e18.5 RIP-Cre;R26R embryos. (D) In $pdx1^{\Delta E2\beta}$ animals, recombined (β-gal$^+$/red) cells no longer express PDX1. (E) In control embryos, proliferation, identified using a phospho-histone H3 antibody (green), is observed in some recombined β cells (red) at e18.5 (arrows). (F) β cells that have lost PDX1 (red cells**)** are not proliferating, although other cells within the same field are (green nuclei).

pancreata from $pdx1^{\Delta E2\beta}$;R26R embryos at e18.5 ($n=3$ animals), indicating that recombination of both the R26R and $pdx1^{flE2}$ allele occurred in the same cells and that any residual Pdx1 protein in these cells is below the level of detection in tissue from late gestation stage embryos (Fig. 2D). Since recombination of both the $pdx1^{flE2}$ and the R262R alleles appears to be coincident, the insulin$^+$ cells present in e17.5–e18.5 mutant pancreata that still contain Pdx1 protein most likely represent newly generated β cells or β cells that are refractory to recombination (Gannon et al., 2000c). Pdx1$^+$/β-gal$^-$ cells were present within pancreata from both control and mutant animals (Figs. 2C, D). These cells likely represent a combination of unrecombined insulin$^+$ cells, other endocrine cell types known to express Pdx1, acinar clusters, and ductal epithelial cells, although acini and ducts usually expressed lower levels of the protein.

Because increased Pdx1 expression has been associated with proliferation of ductal and islet cell populations (Sharma et al., 1999; Song et al., 1999), we examined the effect of β cell *pdx1*

inactivation on proliferation of recombined cells by immunohistochemical detection of phosphorylated histone H3 (pH3), an M-phase-specific marker of actively dividing cells (Gurley et al., 1978). In e18.5 *pdx1*$^{\Delta E2\beta}$ pancreata, β-gal^{+} cells (assumed to be Pdx1^{-}) were always observed to be pH3-negative (Fig. 2F), whereas 8% of β-gal^{+} cells in control pancreata of the same age were observed to be actively proliferating (Fig. 2E). Mutant pancreata did not show a global defect in proliferation, since β-gal^{-} cells were observed to be actively proliferating (Fig. 2F). We conclude that the defect in proliferation was restricted to cells that had undergone RIP-Cre-mediated *pdx1* inactivation and no longer expressed Pdx1 protein.

Loss of pdx1 from β cells causes functional and morphological abnormalities in islets

β cell-specific inactivation of *pdx1* during embryogenesis resulted in very early onset diabetes. As early as postnatal day one (P1), plasma glucose levels during *ad lib* feeding were between 225 and 350 mg/dl in mutant *pdx1*$^{\Delta E2\beta}$ mice ($n=3$), while control littermates had plasma glucose levels of 100–165 mg/dl with a mean of 145 mg/dl ($n=12$). Despite the elevated blood glucose, *pdx1*$^{\Delta E2\beta}$ mice survived past weaning and into adulthood (at least to 8 months, the longest we have maintained the mutant animals).

As adults (1–5 months of age), *pdx1*$^{\Delta E2\beta}$ male mice showed β cell dysfunction when assessed by intra-peritoneal glucose tolerance test (IP-GTT). *pdx1*$^{\Delta E2\beta}$ male mice ($n=5$) had an average fasting blood glucose level of 335 mg/dl (range: 180–475 mg/dl). Upon IP administration of glucose, circulating glucose levels in *pdx1*$^{\Delta E2\beta}$ mice became further elevated and remained high over the test period (Fig. 3A). The average 2-h postprandial glucose measurement was 490 mg/dl. In contrast, control mice ($n=11$) had an average fasting blood glucose level of 98 mg/dl (range: 60–117 mg/dl) and an average 2-h postprandial glucose measurement of 209 mg/dl. Importantly, animals carrying the RIP-Cre transgene alone showed no defects in glucose clearance (data not shown) as has been reported for this transgene on some genetic backgrounds (Lee et al., 2006). *pdx1*$^{\Delta E2\beta}$ male and female mice had dramatically decreased plasma insulin following glucose administration (Fig. 3B). In control mice, plasma insulin levels ranged from 225 to 495 pg/ml 15 min after glucose injection ($n=6$), while plasma insulin in *pdx1*$^{\Delta E2\beta}$ animals ranged from 100 to 170 pg/ml ($n=4$), which is similar to the fasting plasma insulin levels of wild type mice (Fig. 3B). Despite having a reduced number of β cells and reduced plasma insulin levels comparable to males, *pdx1*$^{\Delta E2\beta}$ female animals showed only a mild glucose intolerance relative to control female animals (Fig. 3A). This sexual dimorphism in susceptibility to diabetes has been demonstrated in other rodent models of diabetes (Ostenson et al., 1989; Bell et al., 1994; Zhang et al., 2006).

Confocal immunofluorescence analysis at 1 month of age, when animals are already overtly diabetic, revealed several defects in mutant islets compared with controls (Fig. 4). The numbers of glucagon^{+} and somatostatin^{+} cells were increased (Figs. 4B, D). There was no change in the number of PP cells

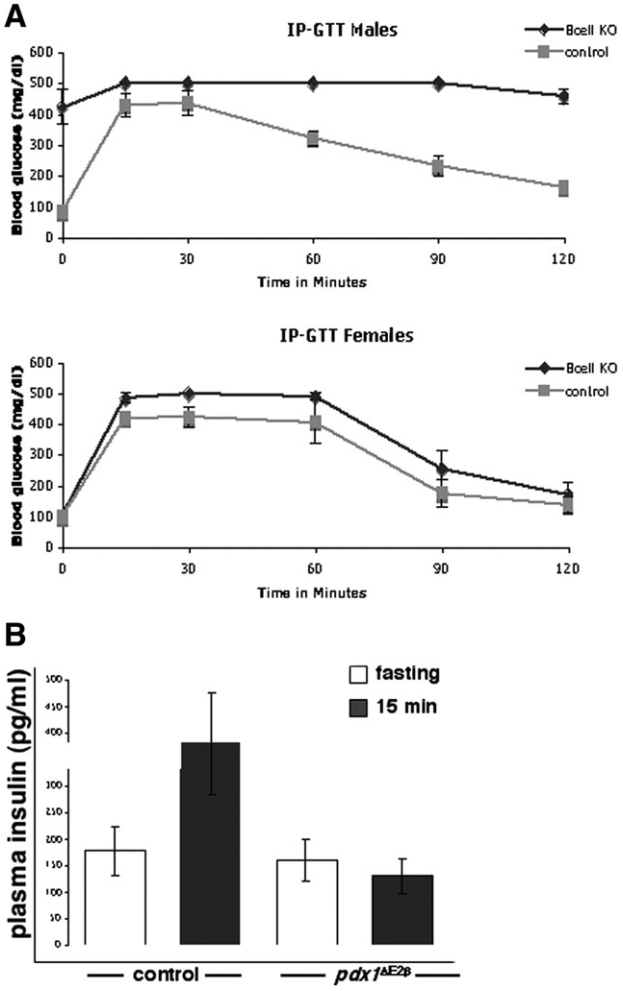

Fig. 3. Early loss of *pdx1* in β cells results in diabetes post-weaning. (A) Male animals (upper panel) in which *pdx1* has been inactivated in β cells (Bcell KO: *pdx1*$^{\Delta E2\beta}$) are diabetic when compared with control littermates. In control animals (gray line), blood glucose levels return to baseline during the course of an IPGTT, while in the *pdx1* β cell knockout animals, blood glucose levels remain elevated throughout the test (black line). Female *pdx1*$^{\Delta E2\beta}$ mice (lower panel) show only a mild impairment in glucose tolerance compared with controls. (B) While control animals show increased plasma insulin levels 15 min after glucose injection (left bars), plasma insulin levels remain at fasting levels in *pdx1*$^{\Delta E2\beta}$ animals (right bars).

(data not shown). We were unsuccessful in our attempts to analyze ghrelin expression using currently available antibodies. Thus, it remains possible that *pdx1*-null β cells are able to convert to ghrelin-expressing cells in islets from *pdx1*$^{\Delta E2\beta}$ animals as observed in other mouse models of embryonic β cell loss (Prado et al., 2004). The excess α and δ cells did not co-express insulin (Fig. 4F and data not shown), in contrast to the study performed by Ahlgren et. al. (1998) in which inactivation of *pdx1* in adult insulin-expressing cells led to large numbers of glucagon/insulin co-expressing cells. In addition, both glucagon^{+} and somatostatin^{+} cells were scattered throughout the islets and not localized to the periphery as in control islets. Third, the level of insulin immunoreactivity in the remaining insulin^{+} cells was reduced, in agreement with the radioimmunoassay data (Fig. 4F).

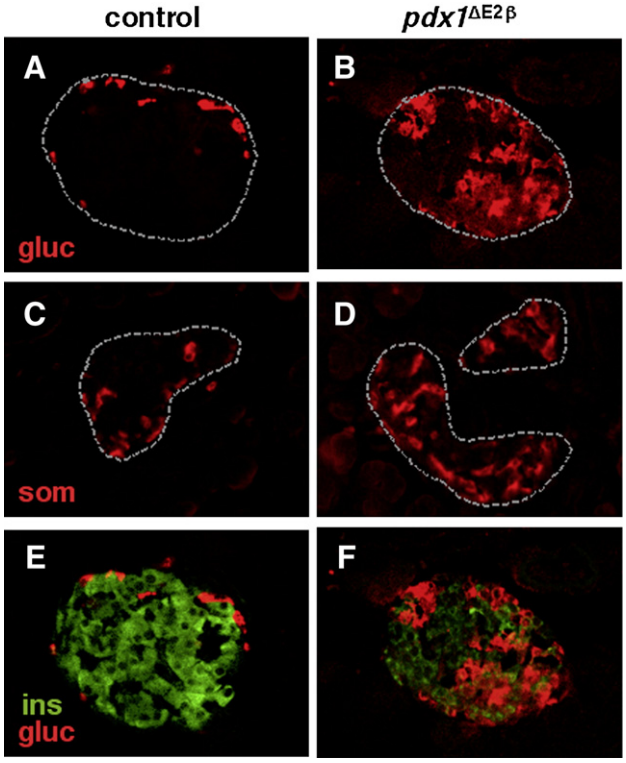

Fig. 4. Loss of *pdx1* in the β cell lineage leads to aberrant islet architecture, and increased numbers of glucagon- and somatostatin-producing cells. Confocal analysis reveals that compared to control animals at 1 month of age (A, C), islets of *pdx1*$^{\Delta E2\beta}$ animals have increased numbers of glucagon^{+} cells (B) and somatostatin^{+} cells (D) that are scattered throughout the islets. *pdx1*$^{\Delta E2\beta}$ animals also have fewer insulin^{+} cells (green) when compared with controls (E, F). Insulin and glucagon are not co-expressed (F).

The lack of insulin co-expression in the excess α and δ cells in our model suggests two possibilities: 1. β→α or δ conversion initiates earlier in development and is complete by the time we examined them at 1 month of age, or, 2. the excess glucagon^{+} and somatostatin^{+} cells we observe in our model do not arise from a cell that previously expressed insulin. The subsequent studies address each of these possibilities.

Expansion of the α cell population in mutant pancreata is observed at e18.5

We used confocal immunofluorescence analysis of pancreata from several stages (gestation, birth, and up to 1 month of age) to determine when the increase in glucagon-expressing cells could be detected, and whether significant numbers of insulin^{+}/glucagon^{+} cells exist at any stage in our mutant pancreata. In contrast to the finding in islets at 1 month of age, there was no significant difference in the number of glucagon^{+} cells between control and *pdx1* β cell knockout pancreata at e14.5 or e17.5 (Figs. 5A and E, and data not shown). The earliest time at which increased numbers of glucagon^{+} cells could be distinguished in mutant pancreata was e18.5 (Figs. 5B and F). By P1, the increased proportion of glucagon^{+} cells was dramatic (compare Figs. 5C, D with G, H). The timing of this increase in glucagon^{+} cells correlated with a severe reduction in the number of insulin-producing cells per islet. In both control and *pdx1*$^{\Delta E2\beta}$ mutant pancreata, clusters of insulin^{+} cells also expressed GLUT2, a marker of second wave β cells, at the membrane at sites of β cell-β cell contacts (data not shown). GLUT2 expression was not detected in insulin^{-} cells.

No glucagon/insulin co-expressing cells were detected at any embryonic time point examined (e14.5, e17.5, e18.5) in either control or mutant pancreata. At P1, very few cells (0.1% of all glucagon^{+} cells; arrowheads in Figs. 5G and H) co-expressing insulin and glucagon were detected within mutant islets, a frequency that was consistent across many tissue sections sampled from several different animals. Thus, we find no evidence for significant numbers of glucagon/insulin co-expressing cells

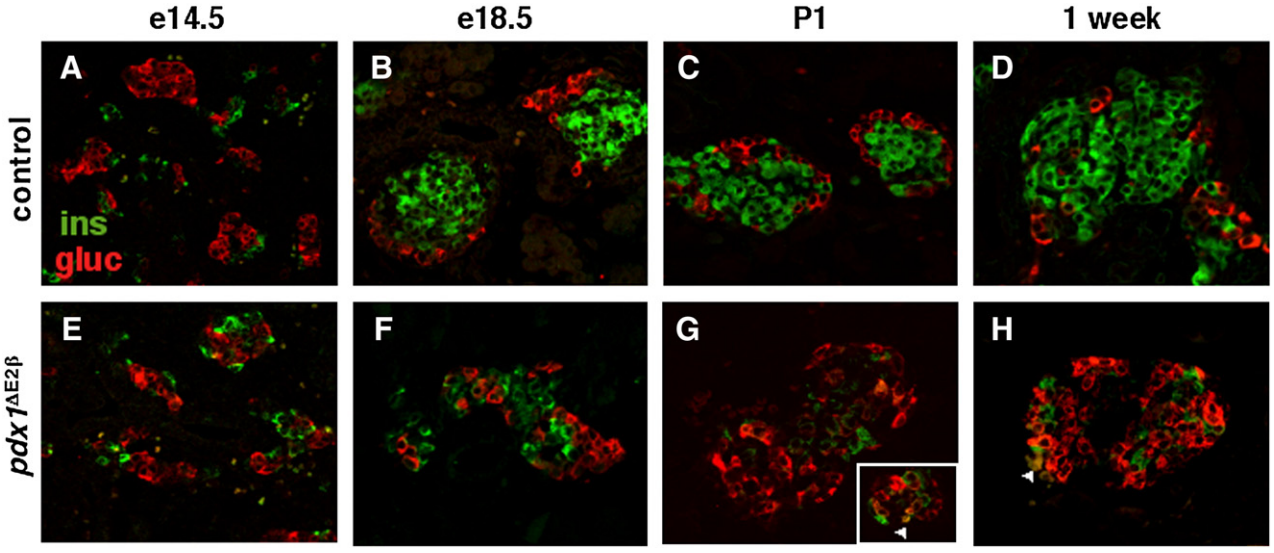

Fig. 5. Alterations in the proportion of α and β cells in *pdx1*$^{\Delta E2\beta}$ animals are first apparent at e18.5. Confocal analysis of insulin (green) and glucagon (red) expression indicates that the proportion of α and β cells is identical in control and *pdx1*$^{\Delta E2\beta}$ embryos at e14.5 (compare panel A with panel E) and e17.5 (not shown). Expansion of the α cell population and decline in the β cell population begins at e18.5 (compare panel B with panel F) and continues into postnatal development (G, H). The vast majority of cells express either one hormone or the other. Cells co-expressing both hormones are occasionally detected (arrowheads in panels G and H).

prior to the expansion of the α cell population, nor at times when this expansion first becomes apparent. These results are inconsistent with the idea that the excess glucagon$^+$ cells arose via a transitional cell type in which insulin-producing cells convert to glucagon-producing cells. This type of analysis, however, cannot exclude the possibility that the β→α transition occurs too rapidly to be detected at the chosen static time points. To determine more directly whether the excess glucagon$^+$ and somatostatin$^+$ cells present within the β cell knockout islets arose from cells that expressed insulin at an earlier time point, we performed lineage-tracing analyses using the R26R reporter mouse.

Excess glucagon$^+$ and somatostatin$^+$ cells do not arise from insulin-producing cells

In RIP-Cre;R26R mice, Cre-mediated β-gal expression is detected specifically in cells that activated the rat insulin promoter transgene. The cell type-independence of the ROSA26 promoter means that progeny of the original cell undergoing recombination stably inherit β-gal expression. Therefore, because the ROSA26 promoter is active in all pancreatic endocrine cell types (Gannon et al., 2000a,c; Kawaguchi et al., 2002), cells derived from insulin-expressing cells should be detectable regardless of their subsequent insulin expression status.

In RIP-Cre;R26R control pancreata from P1 animals, β-gal expression was restricted, as expected, to insulin-producing cells in the islet core (Figs. 6A, C), and absent from glucagon- and somatostatin-producing cells. At P1, >90% of somatostatin$^+$ cells were β-gal$^-$(Fig. 6C), we did detect co-expression of somatostatin and β-gal in ~8% of δ cells (n=12/156) in control pancreata (data not shown). The small percentage of somatostatin$^+$ cells expressing β-gal could reflect leakiness of the RIP-Cre transgene in this lineage, or alternatively, that a proportion of δ cells may arise from an insulin/somatostatin co-expressing intermediate, as suggested previously (Fernandes et al., 1997; Sosa-Pineda et al., 1997). In P1 *pdx1*$^{\Delta E2\beta}$ pancreata, very few β-gal$^+$ cells were detected within the islets (Figs. 6B, D), consistent with the vast decrease in insulin$^+$ cells noted above (e.g. Fig. 5G). Significantly, the β-gal$^+$ cells present at P1 do not co-express either glucagon (0.2%; n=2/849) or somatostatin (0.7%; n=1/142), indicating that the excess α and δ cells did not arise from cells that were previously insulin-producing. Taken together, these data suggest that the increase in glucagon$^+$ and somatostatin$^+$ cells in *pdx1*$^{\Delta E2\beta}$ pancreata is likely caused by increased proliferation of these cell types or their precursors, and not by conversion of *pdx1* null "former-β cells" to other endocrine cell types.

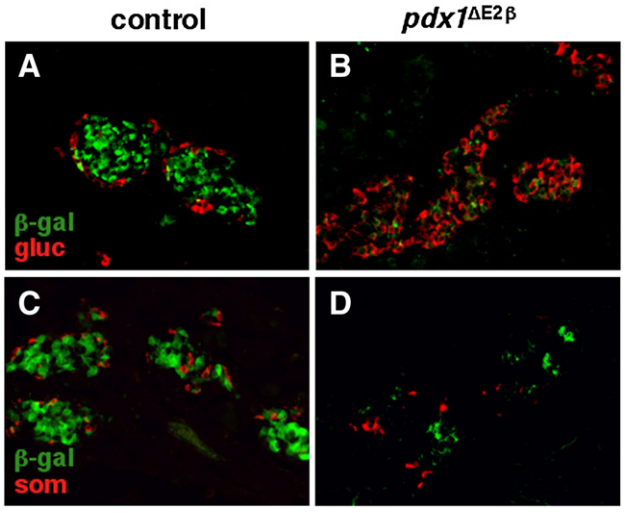

Fig. 6. Lineage tracing analysis at P1 reveals that excess glucagon$^+$ and somatostatin$^+$ cells in *pdx1* β cell knockout animals do not arise from insulin-producing cells. In RIP-Cre;R26R control animals (A, C), insulin-producing cells located at the core of the islet express β-gal (green) as expected. Glucagon$^+$ (A) and somatostatin$^+$ (C) cells (red) are located at the periphery and do not co-express β-gal. In *pdx1*$^{\Delta E2\beta}$ animals, the number of β-gal expressing cells is dramatically reduced (B, C). β-gal expression does not co-localize with glucagon (B) or somatostatin (C) indicating that these cells derive from cells that did not express RIP-Cre.

We next analyzed the proliferation status of differentiated endocrine cells during the second wave of endocrine differentiation (e14.5, e17.5, and e18.5). This developmental window represents the time during which the proportions of the endocrine cell types become abnormal in the conditional mutant animals. pH3-immunopositive cells were scored on at least ten tissue sections containing endocrine cells from two litters at each developmental time point. We failed to detect any difference in proliferation between control (n=4) and mutant (n=2) pancreata at e14.5 (data not shown). Compared with wild type pancreata (n=2), a three-fold increase in pH3-positive glucagon$^+$ cells [2% (n=2/113): wild-type; 6% (n=47/755): mutant] was first detected at e17.5 in β cell knockout pancreata (n=3) and was maintained at e18.5 (Figs. 7A–C and data not shown). The proportion of proliferating insulin$^+$ cells at e17.5 (~1–2%; data not shown) was indistinguishable between control (n=2/196) and mutant (n=7/410) pancreata but, at e18.5, was dramatically reduced in conditional mutant tissue (10% in wild-type n=43/438, 2% in mutants n=12/538; Figs. 7D, E). Proliferation rates in the acinar tissue were not different between control and mutant pancreata. We also examined whether insulin$^+$ cells in mutant pancreata were undergoing apoptosis. TUNEL labeling was performed on control and mutant pancreata from e14.5 and e18.5 embryos, P1 and adult. At no time point did we observe apoptotic insulin$^+$ cells in either control or mutant animals (data not shown).

Discussion

The findings we report here add to our understanding of the continual requirement for *pdx1* in the life history of β cells. The accumulating evidence demonstrates that Pdx1 plays essential regulatory roles both during global pancreas formation and specifically in β cells during embryonic and adult stages. Homozygous null *pdx1* mice have a very early block in pancreas development, but do undergo limited bud outgrowth (Offield et al., 1996). These animals form neither mature islets nor acinar tissue (Jonsson et al., 1994; Offield et al., 1996). Thus, *pdx1* is not required to specify the pancreatic field or to initiate

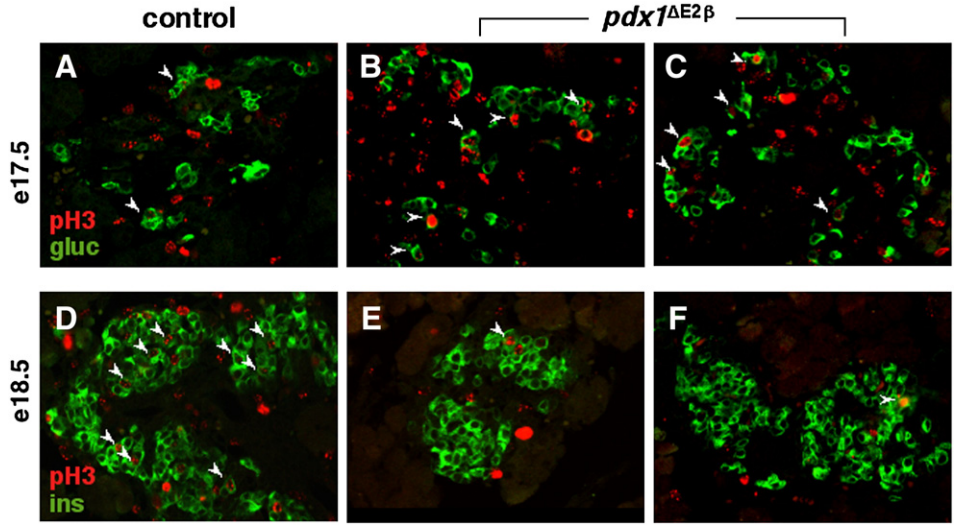

Fig. 7. Loss of *pdx1* in the β cell lineage results in increased proliferation of glucagon$^+$ cells and decreased proliferation of insulin$^+$ cells. Co-labelling of e17.5 and e18.5 pancreatic sections for either glucagon (green, A–C) or insulin (green, D–F) and phospho-histone H3 (red, A–F) reveals that in *pdx1*$^{\Delta E2\beta}$ animals, the fraction of proliferating α cells is increased at e17.5 (compare arrowheads in panel A with panels B, C), while the proportion of insulin-expressing β cells actively proliferating is dramatically decreased at e18.5 (compare arrowheads in panel D with panels E, F). No difference was observed at e14.5 (not shown).

outgrowth from the endodermal epithelium. Loss of *pdx1* function after initial pancreatic bud outgrowth, using a doxycycline-inducible system, results in a block in further pancreas development (Holland et al., 2002), while *pdx1* inactivation at later embryonic stages results in a specific defect in development of the exocrine pancreas (Hale et al., 2005). These studies did not evaluate the effects of *pdx1* removal on second wave endocrine differentiation and expansion. Inactivation of *pdx1* specifically in mature, adult β cells results in diabetes, loss of β cell markers such as Nkx6.1 and GLUT2, and a great decrease in the number of insulin-producing cells (Ahlgren et al., 1998; Holland et al., 2002). Thus, neither the whole animal *pdx1* knockout studies, nor the published conditional inactivation studies address the requirement for *pdx1* specifically during late gestation, the time during which the majority of the insulin-producing cells that will give rise to the mature islet are generated.

pdx1 function in late gestation β cells differs from its function in adult β cells

The current study is the first to define a role for *pdx1* in the late gestation embryonic β cells that are assumed to incorporate into mature islets. In these studies, we used a Cre transgenic line defined via R26R activation for its early and efficient expression in insulin-producing cells within the pancreas. In addition, we included the first lineage-tracing of *pdx1*-deficient cells, and studies of the effect of *pdx1* inactivation on cell proliferation. Mice in which *pdx1* was specifically inactivated in β cells during embryogenesis showed a decrease in proliferation of insulin$^+$ cells at late gestation that translated into a decreased number of β cells. *pdx1*$^{\Delta E2\beta}$ animals showed a concomitant increase in proliferation of glucagon$^+$ cells at late gestation, elevated postprandial blood glucose levels in neonates, and overt diabetes at weaning.

Although both the current study and the one performed by Ahlgren et. al. (1998) used a similar strategy to inactivate *pdx1* in the β cell lineage, Cre-mediated recombination in our study occurred much earlier in the life history of the β cell. Thus, in our experimental system, loss of Pdx1 was observed in a substantial number of insulin-producing cells at e14.5, while a significant decrease in Pdx1 expression was not detected until 3–5 weeks postnatally in the Ahlgren study. This difference in the timing of *pdx1* inactivation is perhaps due to a higher efficiency of recombination driven by our RIP-Cre transgene, or because our experimental design required inactivation of only one floxed *pdx1* allele to reach the null condition. Although it is difficult to rule out, we believe it is less likely that the large difference in timing of *pdx1* inactivation between the two studies is related to differences in the genetic background of the various mice involved.

The fortuitous production of two models of β cell-specific *pdx1* inactivation has therefore revealed specific requirements for *pdx1* in embryonic and adult β cells. In the study by Ahlgren et. al. (1998), *pdx1* inactivation specifically in mature β cells resulted in a reduced number of insulin-producing cells and an associated increase in the number of glucagon-expressing cells, a large fraction of which co-expressed both hormones, leading to the hypothesis that insulin-producing cells were converting to glucagon-producing cells following loss of Pdx1 protein. We also observed a large increase in glucagon$^+$ cells, as well as somatostatin$^+$ cells, following the selective inactivation of *pdx1* in embryonic β cells. This increase in α and δ cell number was apparent as early as e18.5. Lineage tracing of *pdx1* null cells revealed that these excess α and δ cells did not arise from insulin-producing cells. Thus, loss of *pdx1* in late gestation β cells affects these cells differently from loss of *pdx1* in a mature β cell.

The increase in α cell number is associated with increased proliferation of glucagon-producing cells and a decrease in

proliferation of insulin-producing cells. Other studies have shown an association between *pdx1* and proliferation of pancreatic cells (Ahlgren et al., 1996; Sharma et al., 1999; Hart et al., 2000), consistent with a loss of *pdx1* leading to decreased proliferation in the β cell lineage. Intriguingly, the reduction in insulin-producing cells in mutant animals was most dramatic at P1 and 1 week. By 1 month of age, we consistently observed an increase in the number of insulin$^+$ cells (compare Figs. 4 and 5). Because the RIP-Cre transgene used here reproducibly showed activity in approximately 85–90% of β cells at P1 (Gannon et al., 2000c; Sund et al., 2001), it is possible that a population of β cells is refractory to recombination and thus maintains PDX1 expression. These insulin$^+$/Pdx1$^+$ cells likely remain capable of proliferating since β cell mass continues to increase even after weaning via replication of pre-existing β cells (Kaung, 1994; Finegood et al., 1995). Thus, the clusters of insulin$^+$ cells observed in adult mutant islets may represent clonal expansion of unrecombined β cells.

Lineage tracing provides information about the fate of mutant cells

Organogenesis involves the gradual restriction of potential cell fate from multi-potency towards expression of specific differentiated cellular phenotypes. A general regulatory principle for such programs is the sophisticated interplay between cell-autonomous processes and intercellular communication. The current study suggests a previously unappreciated interaction between β cells and other endocrine cells (α and δ) or their progenitors that establishes the appropriate proportions of endocrine cells during islet development. The R26R lineage tracer revealed that the excess glucagon$^+$ or somatostatin$^+$ cells we observe in *pdx1*$^{\Delta E2\beta}$ mutants did not arise from β cells. This finding leads to the idea that the decrease in β cell number results from an increased flux from multipotential or lineage-restricted endocrine progenitors towards the α/δ cell fates, and/or an increased rate of proliferation of differentiated non-β cells in the absence of appropriate numbers of β cells.

All five of the islet endocrine cell types are currently believed to derive from a common multipotential precursor, although confirmation of this hypothesis awaits rigorous testing by lineage-tracing techniques. Regardless, endocrine cells arise from progenitors that at some time in their history expressed *pdx1* and *ngn3* (Gu et al., 2002). The production of the five major endocrine cell types must be regulated in order to consistently produce the proportions seen in normal mature islets. Presumably, environmental cues and lineage-specific combinations of regulatory factors modulate the direction of differentiation of multipotent or lineage-restricted amplifying populations towards one endocrine cell type or another. Very little is known about these instructive processes. Our findings are consistent with a type of feedback effect whereby β cells regulate α cell proliferation in a paracrine fashion (Fig. 8), and we propose a similar mechanism to be responsible for the increased numbers of somatostatin$^+$ cells in β cell-specific *pdx1* knockout animals. A previous study in which insulin-producing cells were specifically ablated using a toxic β cell-specific transgene did not show an effect on the development of α and δ cells (Herrera et al., 1994). In that study, however, endocrine differentiation was assessed only at e16.5, and it is possible, based on our findings, that this is prior to the time at which an effect of β cell loss on α and δ cell development would begin to be apparent. While the nature of the β cell-derived paracrine inhibitory signal is unknown, it is unlikely to be insulin itself since mice lacking both insulin genes show normal numbers of α cells (Duvillie et al., 1997, 2002).

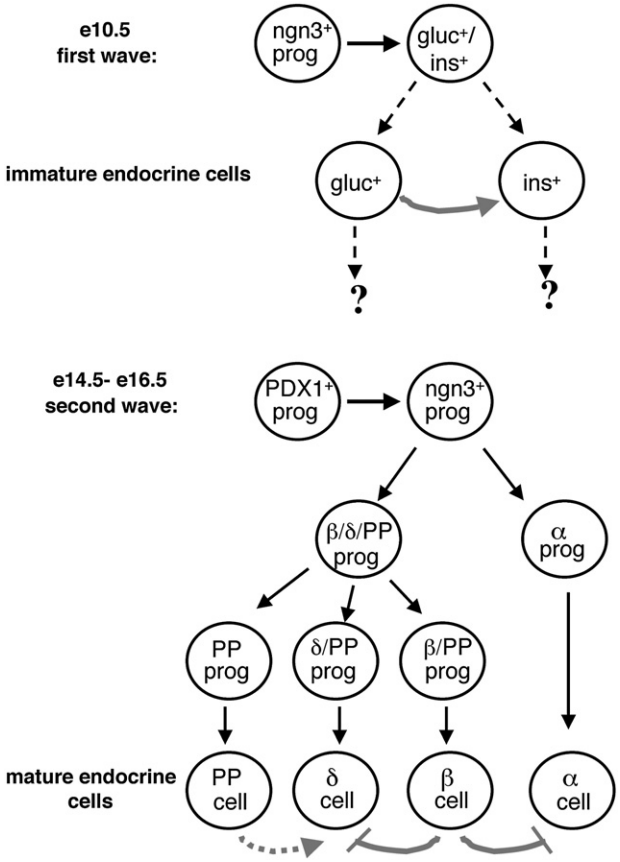

Fig. 8. Model of pancreatic endocrine differentiation and endocrine lineage relationships (solid black lines indicate direct linage relationships, while dashed black lines indicate presumed relationships). Endocrine cells arise from ngn3-expressing progenitors regardless of whether they are generated in the first or second wave of endocrine differentiation. In the first wave, which begins at around e10.5 in the mouse, immature endocrine cells are generated, some of which co-express insulin and glucagon. The fate of these cells is currently unknown, however some studies suggest that the early glucagon$^+$ cells function in the generation of early insulin$^+$ cells (gray arrow). In the second wave of endocrine differentiation, which begins at around e14.5, mature endocrine cells are generated. These cells arise from a PDX1$^+$ cell. The α cell lineage is thought to diverge early, while the existence of a multi-hormone positive cell that gives rise to β, δ, and PP cells has been observed. Mature β cells (and presumably δ cells) arise from a PP-expressing precursor. A paracrine effect of PP cells on δ cell development has not been ruled out (dashed gray arrow). In the current study, we have evidence for an inhibitory effect of β cells on the differentiation/proliferation of α and δ cells (gray lines).

Combining gene inactivation with lineage tracing allows one to determine which, if any, developmental pathways are still open to cells that have lost a particular gene product. For example, the original global inactivation of *Ptf1a* concluded that this gene is required for generation of the exocrine pancreas

(Krapp et al., 1998). Lineage-tracing studies using Cre-mediated R26R activation (Kawaguchi et al., 2002), showed a previously unappreciated expression of *Ptf1a* in precursors of both exocrine and endocrine cells. In homozygous mutants, lineage tracing revealed a conversion of most *Ptf1a*-deficient pancreatic progenitors into normal duodenal cell fates. Similarly, analysis of *Pax6* mutant animals using hormone expression as an indicator of endocrine differentiation led to the conclusion that Pax6 is required for differentiation of α cells and normal numbers of other endocrine cell types (Sander et al., 1997; St-Onge et al., 1997). Conditional inactivation of Pax6 in the pancreas combined with lineage tracing of null mutant cells revealed that normal numbers of endocrine cells actually form in the mutant animals, but that these cells lack certain critical terminal differentiation markers, resulting in impaired α and β cell function (Ashery-Padan et al., 2004).

In addition to lineage-restricted transcriptional regulators (reviewed in (Wilson et al., 2003), it is becoming apparent that pancreatic endocrine cell development depends on interactions and communications between the different islet endocrine lineages (Herrera et al., 1994; Prasadan et al., 2002; Vincent et al., 2003). Additional work is needed to define the nature of the signals produced by the different islet endocrine lineages and how these signals promote differentiation and proliferation to yield fully functional islets.

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